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Inactivation of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase by hypochlorous acid

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Abstract
Myeoloperoxidase catalyses the formation of hypochlorous acid (HOCl) via reaction of H_2O_2 with Cl^- ion. Although HOCl is known to play a major role in the human immune system by killing bacteria and other invading pathogens, excessive generation of this oxidant is known to cause damage to tissue. Recently, it was demonstrated that the control of mitochondrial redox balance and oxidative damage is one of the primary functions of mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) to supply NADPH for antioxidant systems. This study investigated whether the IDPm would be a vulnerable target of HOCl as a purified enzyme and in intact cells. Loss of enzyme activity was observed and the inactivation of IDPm was reversed by thiols. Transfection of HeLa cells with an IDPm small interfering RNA (siRNA) markedly enhanced HOCl-induced oxidative damage to cells. The HOCl-mediated damage to IDPm may result in the perturbation of the cellular antioxidant defense mechanisms and subsequently lead to a pro-oxidant condition.

Keywords: *Hypochlorous acid, antioxidant enzyme, siRNA, redox status*

Introduction

Myeoloperoxidase (MPO) is released by activated neutrophils, monocytes and macrophage-like cells during the respiratory burst that also generates superoxide (O_2^-) and hydrogen peroxide [1,2]. MPO, one of the major granule proteins, catalyses the formation of hypochlorous acid (HOCl) via reaction of H_2O_2 with Cl^- ion [2]. HOCl is known to play a major role in the human immune system by killing bacteria and other invading pathogens [3]. However, excessive or misplaced generation of this oxidant is known to cause damage to tissue [4]. Many *in vitro* studies have shown that HOCl is able to mediate tissue injury and recent work has provided direct evidence for the production of HOCl in various pathological disease states [5]. Proteins are likely to be major targets for reaction with HOCl due to their abundance and high reactivity [6].

Antioxidant enzymes, which provide a substantial defense against damage induced by reactive oxygen species (ROS), could be susceptible to the damaging effect of HOCl. It is implied that the inactivation of antioxidant enzymes by HOCl may lead to the perturbation of the cellular antioxidant defense system and subsequently exacerbate the harmful effect of HOCl as well as ROS. The isocitrate dehydrogenases (ICDHs; EC1.1.1.41 and EC1.1.1.42) catalyse oxidative decarboxylation of isocitrate to α-ketoglutarate and require either NAD^+ or $NADP^+$, producing NADH and NADPH, respectively [7]. NADPH is an essential reducing equivalent for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of NADPH-dependent thioredoxin

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system [8,9], both are important in the protection of cells from oxidative damage. Therefore, mitochondrial ICDH (IDPm) may play an antioxidant role during oxidative stress. We recently reported that IDPm is involved in the supply of NADPH needed for GSH production against mitochondrial oxidative damage [10]. Hence, the damage of IDPm may result in the perturbation of the balance between oxidants and antioxidants and subsequently lead to a pro-oxidant condition. Since cysteine residues serve as an essential role in the catalytic function of IDPm [11,12], the highly reactive sulphydryl groups in IDPm could be potential targets of HOCl.

In the present study, we have evaluated the susceptibility of IDPm as a purified enzyme and in HeLa cells to HOCl. The role of IDPm in cellular defense against HOCl-induced oxidative injury was investigated with HeLa cells transfected with IDPm small interfering RNA (siRNA). The data indicate that the HOCl-mediated damage to IDPm may result in the perturbation of cellular antioxidant mechanisms and subsequently lead to a pro-oxidant condition.

Materials and methods

Materials

β-$NADP^+$, isocitrate, L-buthionine-(*S,R*)-sulphoximine (BSO), IDPm from pig heart, NaOCl and anti-rabbit IgG tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123, 2′,7′-dichlorofluoroscin diacetate (DCFH-DA), t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC) and rhodamine 123 were purchased from Molecular Probes (Eugene, OR). Electrophoresis reagents and protein assay kit were purchased from Bio-Rad (Hercules, CA). Antibodies were purchased from Santa Cruz (Santa Cruz, CA) or Cell Signaling (Beverly, MA). A peptide representing the N-terminal 16 amino acids of mouse IDPm (ADKRIKVAKPVVEMPG) was used to prepare polyclonal anti-IDPm antibodies [10].

Cell culture and cytotoxicity

HeLa cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM glutamine and 100 units/ml penicillin/streptomycin. The cells were incubated at 37°C in a 90% humidified atmosphere containing 5% CO_2. Cytotoxicity after treatment with various concentrations of HOCl for 1 h was assessed by MTT assay [10]. For GSH depletion, the cells were incubated for 24 h with 1 mM BSO, as earlier described by Ghibelli et al. [13].

Knockdown of IDPm by siRNA

IDPm siRNA and control (scrambled) siRNA were purchased from Samchully Pharm (Seoul, Korea). The sequences of the dsRNAs of IDPm and control used in the current experiments are as follows. For IDPm, sense and antisense siRNAs are 5′-AGACC-GACUUCGACAAGAAdTdT-3′ and 5′-UUCUU-GUCGAAGUCGGUCUdTdT-3′, respectively. For scrambled control, sense and antisense siRNAs are 5′-CUGAUGACCUGAGUGAAUGdTdT-3′ and 5′-CAUUCACUCAGGUCAUCAGdTdT-3′, respectively. HeLa cells were transfected with 60 nM oligonucleotide by using Lipofectamine (Invitrogen) in serum-free conditions according to the manufacturer's protocol. After incubation for 2 days, the cells were washed and supplemented with fresh medium containing 10% FBS.

IDPm activity assay

IDPm (6.5 μg) was added to 1 ml Tris buffer, pH 7.4, containing $NADP^+$ (2 mM), $MgCl_2$ (2 mM) and isocitrate (5 mM). Activity of IDPm was measured by the production of NADPH at 340 nm at 25°C [10]. One unit of IDPm activity is defined as the amount of enzyme catalysing the production of 1 μmol of NADPH/min. For the determination of IDPm activity in HeLa cells, cells were collected at 1000 × g for 10 min at 4°C and were washed once with cold PBS. Briefly, cells were homogenized with a Dounce homogenizer in sucrose buffer (0.32 M sucrose, 10 mM Tris-Cl, pH 7.4). Cell homogenate was centrifuged at 1000 × g for 5 min and the supernatant was further centrifuged at 15 000 × g for 30 min. The precipitates were washed twice with sucrose buffer to collect mitochondria pellet. The mitochondrial pellets were resuspended in 1X PBS containing 0.1% Triton-X100, disrupted by ultrasonication (4710 Series, Cole-Palmer, Chicago, IL) twice at 40% of maximum setting for 10 s and centrifuged at 15 000 × g for 30 min. The supernatant was used to measure the activity of IDPm. The protein levels were determined by the method of Bradford using reagents purchased from Bio-Rad.

Immunoblot analysis

Proteins were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membranes and subsequently subjected to immunoblot analysis using anti-IDPm antibodies. Immunoreactive antigen was then recognized by using horseradish peroxidase-labelled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Cellular redox status and oxidative damage

Intracellular peroxide production was measured using the oxidant-sensitive fluorescent probe DCFH-DA with confocal microscopy [10]. The intracellular GSH level was determined by using a GSH-sensitive fluorescence dye CMAC. HeLa cells (1×10^6 cells/ml) were incubated with 5 μM CMAC cell tracker for 30 min. The images of CMAC cell tracker fluorescence by GSH was analysed by the Zeiss Axiovert 200 inverted microscope at fluorescence DAPI region (excitation, 351 nm; emission, 380 nm) [14]. Thiobarbituric acid-reactive substances (TBARS) were determined as an independent measurement of lipid peroxidation as described [10]. 8-OH-dG levels of HeLa cells were estimated by using a fluorescent binding assay as described by Struthers et al. [15]. After HeLa cells were exposed to HOCl, cells were fixed and permeabilized with ice-cold methanol for 15 min. DNA damage was visualized with avidin-conjugated TRITC (1:200 in PBS for 1 h) for fluorescent microscopy with 488 nm excitation and 580 nm emission.

Mitochondrial redox status and damage

To evaluate the levels of mitochondrial ROS cells in PBS were incubated for 20 min at 37°C with 5 μM DHR 123 and cells loaded with the fluorescent probes were imaged with a fluorescence microscope. Mitochondrial membrane permeability transition (MPT) was measured by the incorporation of rhodamine 123 dye into the mitochondria, as previously described [16]. Cells were exposed to anti-cancer agents and then treated with 5 μM rhodamine 123 for 15 min and excited at 488 nm with an argon laser.

Quantitation of relative fluorescence

The averages of fluorescence intensity from fluorescence images were calculated as described [17].

Statistical analysis

The difference between two mean values was analysed by Student's *t*-test and was considered to be statistically significant when $p < 0.05$.

Replicates

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

Incubation of IDPm with HOCl resulted in a concentration-dependent loss of enzyme activity as shown in Figure 1A. In order to gain insight into the mechanism of IDPm damage by HOCl, IDPm was allowed to react simultaneously with HOCl and thiols such as DTT, cysteine and GSH. As shown in Figure 1A, IDPm was protected from HOCl by co-treatment of thiols, suggesting that HOCl reacts to cysteine residue(s) of IDPm which presumably induce inactivation of the enzyme. To evaluate the protective effect of substrates on the inactivation of IDPm by HOCl, IDPm was incubated with HOCl in the presence of NADP$^+$ or isocitrate for 1 h at 37°C. Although the incubation of IDPm with 40 μM HOCl resulted in 62% inhibition of IDPm activity, the addition of 1 mM NADP$^+$ or 4 mM isocitrate protected IDPm from inactivation (Figure 1B). The protective effect of substrates supports the suggestion that HOCl reacts with cysteine residue(s) residing in the active site. To test whether cellular IDPm was inactivated, HeLa cells were treated with various concentrations of HOCl for 1 h. As shown in Figure 1C, IDPm inactivation in HeLa cells requires higher concentrations of HOCl when compared to the purified enzyme. This apparent difference in the inhibitory effects *in situ* and *in vitro* can reasonably be attributed to differences in the conditions of the two experiments. In particular, cellular reducing agents may protect HOCl-induced inactivation of IDPm *in situ*. Thus, the *in situ* experiment probably reflects an under-estimation of the actual sensitivity of IDPm to the inactivation by HOCl. In order to support that cysteine residues of IDPm are targets for HOCl, intracellular GSH was depleted by BSO, a specific inhibitor of γ-glutamylcysteine synthetase. As shown in Figure 1D, the prolongled incubation with 1 mM BSO for 24 h significantly enhanced HOCl-induced reduction of activity of IDPm in HeLa cells.

The silencing of gene expression by siRNA duplexes has proved to be a powerful tool for the study of gene functions in mammalian cells. We have used *in vitro*-transcribed siRNAs specific for the mRNA of human IDPm for the transient transfection of HeLa cells. After transfection, the cells were assayed for IDPm protein expression by immunoblotting assay. IDPm siRNA was effective in decreasing protein levels and IDPm activity (Figure 2A). When cultured HeLa cells were treated with HOCl, a concentration-dependent decrease in cell viability was observed. However, HeLa cells transfected with IDPm siRNA were significantly more sensitive than control cells transfected with scrambled siRNA (Figure 2B). The change of morphology induced by HOCl was more pronounced in IDPm siRNA-transfected HeLa cells compared to control cells (Figure 2C).

To investigate whether the difference in cytotoxicity of control and IDPm siRNA-transfected cells upon exposure to HOCl is associated with ROS formation, the levels of intracellular peroxides in HeLa cells were evaluated by a confocal microscope with the oxidant-sensitive probe DCFH-DA. As

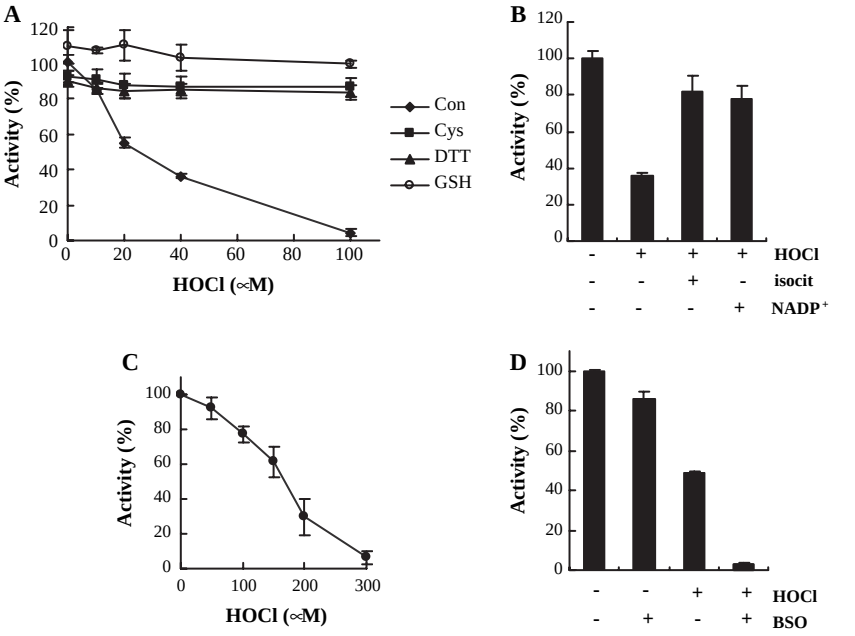

Figure 1. Inactivation of IDPm by HOCl. (A) IDPm was incubated with various concentrations of HOCl for 1 h with and without 1 mM GSH, 1 mM cysteine or 1 mM DTT. IDPm activity was measured under standard assay conditions. Activities are given as a percentage of the control value. Data are presented as means ± SD of three separate experiments. (B) Effect of substrates on the inactivation of IDPm by HOCl. After incubation of IDPm with 40 μM HOCl in the presence of 4 mM isocitrate or 1 mM $NADP^+$ for 1 h, activities of IDPm were determined. Data are presented as means ± SD of three separate experiments. (C) Inactivation of IDPm in HOCl-treated HeLa cells. HeLa cells were incubated with various concentrations of HOCl for 1 h and disrupted by sonication. (D) Inactivation of IDPm in BSO-pre-treated (1 mM, 24 h) HeLa cells exposed to 150 μM HOCl for 1 h. The mitochondrial fraction was prepared and activity of IDPm was determined. Activities are given as a percentage of the control value. Data are presented as means ± SD of three separate experiments.

shown in Figure 3A, an increase in DCF fluorescence was observed in HeLa cells when they were exposed to HOCl and the increase of fluorescence was significantly enhanced in IDPm siRNA-transfected cells. GSH is one of the most abundant intracellular antioxidants and determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. Cellular GSH levels determined with the GSH-sensitive fluorescent dye CMAC in HeLa cells treated with 150 μM HOCl were decreased significantly in IDPm siRNA-transfected HeLa cells compared to control cells (Figure 3B). Lipid peroxidation and the fluorescent intensity which reflects the endogenous levels of 8-OH-dG in DNA were significantly increased in IDPm siRNA-transfected HeLa cells compared to control cells upon exposure to HOCl (Figure 3C and D). Taken together, these results indicate that

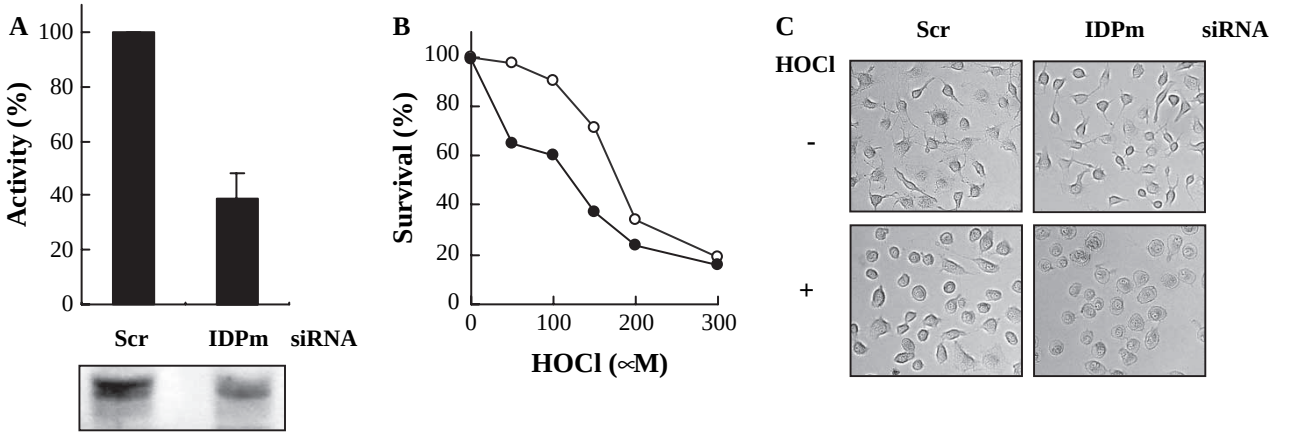

Figure 2. Knockdown of IDPm by siRNA in HeLa cells and its effect on cytotioxicity. (A) HeLa cells were transfected with scrambled siRNA (Scr) or IDPm siRNA. After 48 h, the transfected cells were disrupted by sonication and then the activity and the protein level of IDPm were determined. Data are presented as means ± SD of three separate experiments. (B) Effect of siRNA on cytotoxicity of HOCl in HeLa cells. HeLa cells transfected with scrambled siRNA (open circles) or IDPm siRNA (closed circles) were treated with various concentrations of HOCl for 1 h and cell viability was determined using MTT assay. (C) The morphological changes of the HOCl-treated (150 μM, 1 h) HeLa transfectant cells were analysed by microscopy. Scr, scrambled siRNA.

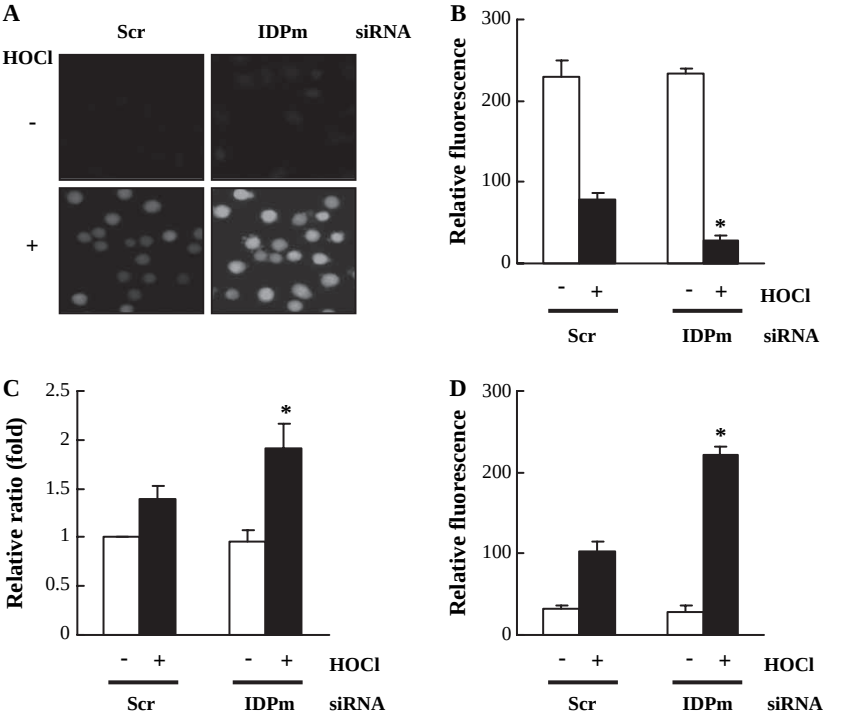

Figure 3. Effects of IDPm siRNA on the cellular redox status and oxidative damage in HeLa cells exposed to 150 μM HOCl for 1 h. (A) Measurement of *in vivo* molecular oxidation. DCF fluorescence was measured in HeLa transfectant cells exposed to HOCl by confocal microscopy. (B) GSH levels reflected by fluorescence images of CMAC-loaded cells were obtained under microscopy. The averages of fluorescence intensity were calculated as described [17]. Data are presented as means ± SD of five separate experiments. * $p < 0.01$ vs scrambled siRNA transfected cells exposed to HOCl. (C) Lipid peroxidation of HeLa cells after exposure to HOCl. The levels of MDA accumulated in the cells were determined by using a TBARS assay. Data are presented as means ± SD of three separate experiments. * $p <$ 0.01 vs scrambled siRNA transfected cells exposed to HOCl. (D) 8-OH-dG levels in HeLa transfectant cells reflected by the binding of avidin-TRITC were analysed. Fluorescence images were obtained under microscopy. The averages of fluorescence intensity were calculated as described [17]. Data are presented as means ± SD of three separate experiments. * $p < 0.01$ vs scrambled siRNA transfected cells exposed to HOCl. Scr, scrambled siRNA.

decreased activity of IDPm by siRNA enhances HOCl-induced cellular damage.

Alterations in mitochondrial integrity and function may play an important role in cell death. MPT, associated with the opening of large pores in the mitochondrial membranes, is a very important event in cell death and ROS is one of the major stimuli that change MPT [18]. To answer whether IDPm modulates the MPT upon exposure to HOCl, we determined the change in MPT by intensity of fluorescence emitting from a lipophilic cation dye, rhodamine 123. High fluorescence represents high mirochondrial membrane potential ($\triangle\psi$m), which correlates with healthy mitochondria. Significantly less rhodamine 123 dye was taken up by the mitochondria of IDPm siRNA-transfected cells, compared with control cells (Figure 4A). To determine if changes in MPT were accompanied by changes in intracellular ROS, the levels of intracellular peroxides in the mitochondria of HeLa cells were evaluated by a confocal microscope with the oxidant-sensitive probe DHR 123. As shown in Figure 4B, the intensity of fluorescence was significantly lower in control cells when compared to that in the mitochondria of IDPm siRNA-transfected cells when HeLa cells were exposed to HOCl.

Discussion

HOCl is a powerful oxidizing agent that can react with many biological molecules. Cysteine and methionine residues of proteins are the most readily oxidized [6]. It has been established that IDPm contains reduced cysteinyl residues that are important for enzyme activity [11,12]. The sulphydryl groups of IDPm are susceptible to modification by ROS and reactive nitrogen species [19–21]. Here we present evidence indicating that IDPm can be inhibited by HOCl, presumably through modification by its cysteine residues. Because IDPm inactivation was prevented by adding its substrates, $NADP^+$ and isocitrate, we conclude that target sites for HOCl are likely to include cysteine residue near the substrate binding site.

NADPH is an essential co-factor for the regeneration of GSH, the most abundant low-molecular-mass thiol in most organisms, by glutathione reductase in addition to its critical role for the activity of

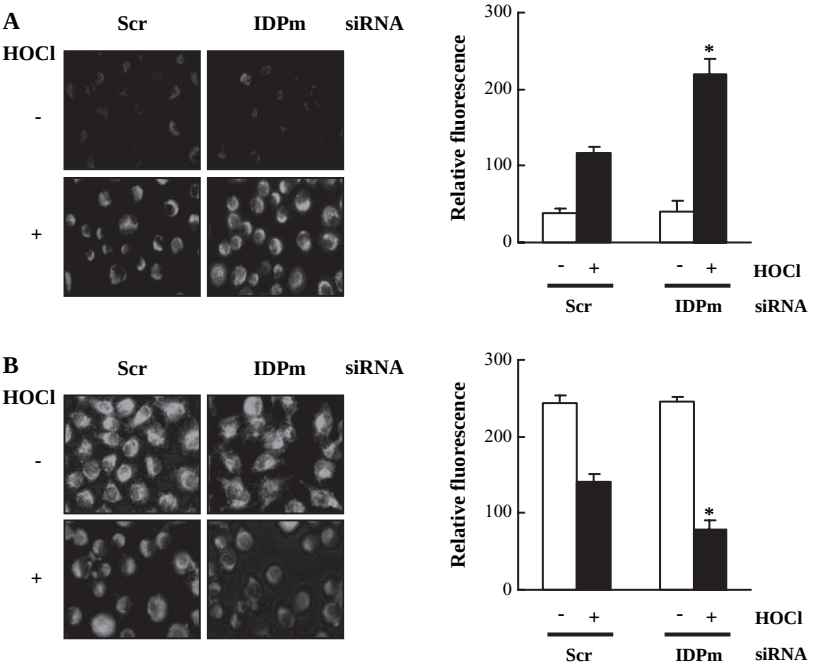

Figure 4. Effects of IDPm siRNA on mitochondrial dysfunction and mitochondrial redox status of HeLa transfectant cells exposed to 150 μM HOCl for 1 h. (A) Effect of IDPm on MPT. MPT of HeLa transfectant cells was measured by the incorporation of rhodamine 123 dye into the mitochondria. (B) Effect of IDPm on mitochondrial ROS generation. DHR 123 was employed to detect mitochondrial ROS. Fluorescence images were obtained under microscopy (left) and the averages of fluorescence intensity (right) were calculated as described [17]. Data are presented as means ± SD of five separate experiments. * $p < 0.01$ vs scrambled siRNA transfected cells exposed to HOCl. Scr, scrambled siRNA.

NADPH-dependent thioredoxin system [8,9]. IDPm is a key enzyme in cellular defense against oxidative damage by supplying NADPH in the mitochondria, needed for the regeneration of mitochondrial GSH or thioredoxin. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppressed the oxidative stress and concomitant ROS-mediated damage. It is well established that mitochondrial dysfunction is directly and indirectly involved in a variety of pathological states caused by genetic mutations as well as exogenous compounds or agents [22]. Mitochondrial GSH becomes critically important against ROS-mediated damage because it not only functions as a potent antioxidant but is also required for the activities of mitochondrial glutathione peroxidase and mitochondrial phospholipid hydroperoxide glutathione peroxidase [23], which removes mitochondrial peroxides. NADPH is a major source of reducing equivalents and cofactors for mitochondrial thioredoxin peroxidase family/peroxiredoxin family including peroxiredoxin III/protein SP-22 [24–26] and peroxiredoxin V/AOEB166 [27]. Therefore, any mitochondrial NADPH producer, if present, becomes critically important for cellular defense against ROS-mediated damage. In this regard, the inactivation of IDPm by HOCl may result in the disruption in regulating the mitochondrial redox balance by providing NADPH.

In order to elucidate the role of IDPm, the effect of IDPm siRNA transfection on HeLa cells exposed to HOCl was evaluated in regards to modulation of survival, cellular redox status and mitochondrial dysfunction. The suppression of IDPm expression by siRNA significantly increased ROS level and enhanced the cellular oxidative damage. Mitochondria are vulnerable to oxidants because they are the major source of free radicals in the cells and are limited in their ability to cope with oxidative stress [28]. It is well established that mitochondrial dysfunction is directly and indirectly involved in cell death and a variety of pathological states [29]. The changes caused by HOCl are compatible with mitochondrial failure, generation of ROS and accumulation of rhodamine 123 which reflect mitochondrial swelling or changes in the mitochondrial inner membrane. A clear enhancement of such damages in IDPm siRNA-transfected cells compared to control cells exacerbates a deterioration of bioenergetic state.

In conclusion, the HOCl-mediated damage to IDPm may result in the perturbation of cellular antioxidant defense mechanisms and subsequently lead to a pro-oxidant condition.

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